METHODS FOR LARGE SCALE PRODUCTION OF RECOMBINANT DNA-DERIVED TPA OR K2S MOLECULES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 60/268,574, filed February 15, 2001, and to Great Britain Patent Application No. GB 00 27 779.8, filed November 14, 2000. The full disclosure of each of these applications is herein incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] The invention belongs to the field of thrombolysis and of tissue plasminogen activator (tPA) derivative production in prokaryotic cells.

[0003] The invention relates to methods for the production of a recombinant DNA-derived tPA, a variant therof or a (Kringle 2 Serine) K2S molecule or a variant therof in prokaryotic cells, wherein said tPA or K2S or variant is secreted extracellularly as an active and correctly folded protein, and the prokaryotic cell contains and expresses a vector comprising the DNA coding for said tPA or K2S or variant operably linked to the DNA coding for the signal peptide OmpA. The invention further relates to specific K2S derivatives obtainable by said method. The invention further relates to said DNA molecules and the use of said DNA molecules in said methods.

Related Art

[0004] Tissue plasminogen activator (tPA) is a polypeptide containing 527 amino acid residues (Pennica, D., *et al., Nature 301*:214-221 (1983)) with a molecular mass of 72 kDa. The molecule is divided into five structural domains. Nearby the N-terminal region is a looped finger domain, which is followed by a growth factor domain. Two similar domains, kringle 1 and kringle 2, are following. Both finger and kringle 2 domains bind specifically to

the fibrin clots thereby accelerating tPA protein activation of bound plasminogen. Downstream of kringle 2 is the serine protease, with its catalytic site located at the C-terminus. The serine protease is responsible for converting plasminogen to plasmin a reaction important in the homeostasis of fibrin formation and clot dissolution. The correct folding of tPA requires the correct pairing of 17 disulfide bridges in the molecule (Allen, S., *et al., J. Biol. Chem. 270*:4797-4804 (1995)).

[0005] Clinically, tPA is a thrombolytic agent of choice for the treatment of acute myocardial infarction, pulmonary embolism, stroke, peripheral arterial occlusions, and other thromboembolic diseases. It has the advantage of causing no side effects on systemic haemorrhaging and fibrinogen depletion (Camiolo, S. M., *et al., Proc. Soc. Exp. Biol. Med. 38*:277-280 (1971)). Bowes melanoma cells were first used as a source in tPA production for therapeutic purposes (Griffiths, J. B. and Electricwala, A., *Adv. Biochem. Eng. Biotechnol. 34*:147-166 (1987)). Since a consistent process with efficient production of highly purified protein in good yield is required for clinical use, the construction of full-length recombinant-tPA (r-tPA) progressed to mammalian cells. Chinese hamster ovary cells were transfected with the tPA gene to synthesize the r-tPA (Cartwright, T., "Production of t-PA from animal cell culture," in *Animal Cell Biotechnology, Vol 5*, Spier and Griffiths eds., Academic Press, New York, NY (1992), pp217-245; Lubiniecki, A., *et al.*, "Selected strategies for manufacture and control of recombinant tissue plasminogen activator prepared from cell culture," in Spier, *et al.*, eds., *Advances In Animal Cell Biology And Technology For Bioprocesses*, Butterworths, London, p. 442-451). The recombinant DNA derived product produced by a mammalian cell culture fermentation system is harvested and purified from the culture medium. Attracted by simplicity and economy of production, a number of efforts in producing r-tPA from microorganisms, expecially bacteria, and more especially from *Escherichia coli*, were investigated (Datar, R. V., *et al., Biotechnology 11*:349-357 (1993); Harris, T. J., *et al., Mol. Biol. Med. 3*:279-292 (1986); Sarmientos, P., *et al.,*

Biotechnology 7:495-501 (1989)). Regarding the low yield and the formation of inclusion bodies, which resulted in misfolding and in an inactive enzyme, numerous strategies have been proposed to overcome these problems.

[0006] Several deletion-mutant variants including kringle 2 plus serine protease (*K2S*) were considered. However, the enzymatic activity of the recombinant-K2S (r-K2S) was obtained only when refolding processes of purified inclusion bodies from cytoplasmic compartment were achieved (Hu, C. K., *et al., Biochemistry 33*:11760-11766 (1994); Saito, Y., *et al., Biotechnol. Prog. 10*:472-479 (1994)). In order to avoid the cumbersome refolding processes, impurities of misfolded proteins, and periplasmic protein delivery, special bacterial expression systems were exploited (Betton, J. M., *et al., J. Biol. Chem. 273*:8897-8902 (1998); Scherrer, S., *et al., Appl. Microbiol. Biotechnol. 42*:85-89 (1994)). Despite periplasmic expression of tPA, overexpression led to inactive aggregates, even in the relatively high oxidizing condition in the periplasm.

[0007] In the prior art, there are a few descriptions of methods for the preparation of recombinant K2S in *E. coli*. However, there is no disclosure of a method leading to a cost effective method for large scale production of biologically active K2S.

[0008] Obukowicz *et al.* (Obukowicz, M. G., *et al., Biochemistry 29*:9737-9745 (1990)) expressed and purified r-K2S from periplasmic space. The obvious disadvantage of this method was an extra periplasmic extraction step, which is not suitable for large scale production.

[0009] Saito *et al.* (Saito, Y., *et al., Biotechnol. Prog. 10*:472-479 (1994)) disclose the cytoplasmic expression of r-K2S. The authors used an *in vivo* renaturation processes for the expressed r-K2S, which was purified from the cytoplasmic space of *E. coli* as inclusion body. Boehringer Mannheim use a similar cumbersome denaturing/refolding process involving the steps of cell digestion, solubilization under denaturing and reducing conditions and reactivation under oxidizing conditions in the presence of GSH/GSSG which is not cost effective (Martin, U., *et al., Kardiol. 79*:167-170 (1990)) and

requires mutation of the amino acid sequence with possibly antigenic potential.

[0010] In 1991, Waldenström *et al.* (Waldenström, M., *et al., Gene 99*:243-248 (1991)) constructed a vector (pEZZK2P) for the secretion of kringle 2 plus serine protease domain to *E. coli* culture supernatant. Hydroxylamine was used to remove the ZZ fusion peptide from IgG-Sepharose purified fraction. The cleavage agent hydroxylamine required modification of the cleavage sites of kringle 2 plus serine protease (Asn177 → Ser and Asn184 → Gln) thus to protect it from hydroxylamine digestion. However, the resulting non-native, not properly folded K2S molecule is not suitable for therapeutic purposes. No enzymatic activity regarding fibrin binding/protease activity was disclosed. The unusual sequence may even activate the human immune system.

SUMMARY OF THE INVENTION

[0011] The problem underlying the present invention was thus to provide a commercially applicable method for large scale production of tPA molecules and derivatives therof, e.g. K2S, wherein the K2S molecule is secreted in its biologically active form into the culture supernatant. The problem was solved within the scope of the claims and specification of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0012] FIG. 1. Validation of PCR amplification product of the K2S gene from the p51-3 vector by using SK2/174 and ASSP primers. Lane 1 shows 1 kb marker (Roche Molecular Biochemicals, Indianapolis, IN). Lane 2 was loaded with 1 μl of amplified product. A single band at 1110 bp is depicted. The electrophoresis was performed on a 1% agarose gel.

[0013] FIG. 2. Identification of inserted K2S gene at 1110 bp (*) after Sfi I digested pComb3H-K2S was demonstrated in lane 3. Lane 1 shows 1 kb marker. Lane 2 was loaded with uncut pComb3H-K2S. The electrophoresis was performed on a 1% agarose gel.

[0014] FIG. 3. Scheme of pComb3H-K2S showing two Sfi I cloning sites into which the K2S gene was inserted. Signal sequence (OmpA), ribosome binding site (RIBS), lac promotor, and gpIII gene are also depicted.

[0015] FIG. 4. Schematic diagram of the mutation site at the junction between the K2S and gpIII genes on pComb3H-K2S. The annealing site of pComb3H-K2S is bound with a set of mutation primers (MSTPA and MASTPA) containing modified oligonucleosides (underlined). After performing the cycle amplification, the Sfi I site 1 (in bold) is modified and lost in the newly synthesized strand.

[0016] FIG. 5. Characterization of newly synthesized MpComb3H-K2S by the Sfi I restriction enzyme. A single band at 4319 bp that refers to a single cleavage site of MpComb3H-K2S is observed in lane 3. No inserted K2S band at 1110 bp can be visualized. Lane 1 shows 1 kb marker. Lane 2 was loaded with uncut MpComb3H-K2S. The electrophoresis was performed on a 1% agarose gel.

[0017] FIG. 6. Identification of immunological reactive band with of recombinant DNA-derived protein purified from XM[K2S] culture supernatant with sheep anti-tPA conjugated HRP. Lane 1 was loaded with 40 ng of standard melanoma tPA (86/670), which showed the reactive band at 70 kDa. The partially purified and concentrated culture supernatants from non-transformed *E. coli* XL1- Blue and XM[K2S] were applied to lane 2 and 3 respectively. The distinct reactive band was particularly demonstrated in lane 3 at 39 kDa.

[0018] FIG. 7. Molecular weight determination of extracellular r-K2S harboring active serine protease domain by copolymerized plasminogen polyacrylamide gel electrophoresis. Lane 1 contained the indicated molecular weight standards ($\times 10^{-3}$), SDS-6H (Sigma, Saint Louis, MO). Fifty μg of the 55% saturated ammonium sulfate precipitated culture supernatant of XL-1 Blue, Xl-1 Blue transformed with pComb3HSS, and XM[K2S] were loaded in lane 2, 3, and 4 respectively. Lane 5 contained 50 mIU of standard melanoma tPA (86/670). Transparent zones of digested plasminogen in polyacrylamide

gel are visible only in lane 4 at molecular weight of 34 and 37 kDa (B) and lane 5 at molecular weight of 66 and 72 kDa (A).

[0019] FIG. 8. Structure A (SEQ ID NO:11) Native K2S molecule from amino acids 174-527 without modification.

[0020] FIG. 9. Structure B-0 (SEQ ID NO:12) Native K2S molecule from amino acids 197-527 without modification.

[0021] FIG. 10. Structure B-1 (SEQ ID NO:13) K2S molecule from amino acids 193-527, wherein to Structure B-0 of Fig. 9 the amino acids SEGN were added at the N-terminal portion.

[0022] FIG. 11. Structure B-2 (SEQ ID NO:14) K2S molecule from amino acids 193-527, as in Fig. 10, wherein Cys-261 was exchanged for Ser.

[0023] FIG. 12. Structure B-3 (SEQ ID NO:15) K2S molecule from amino acids 191-527, wherein to Structure B-0 of Fig. 9 the amino acids SEGNSD were added at the N-terminal portion.

[0024] FIG. 13. Structure B-4 (SEQ ID NO:16) K2S molecule from amino acids 191-527, as in Fig. 12, wherein Cys-261 was exchanged for Ser.

[0025] FIG. 14. Structure C (SEQ ID NO:17) Native K2S molecule from amino acids 220-527 without modification. This molecule may be further modified in a similar manner as disclosed for structure B in figures 10-13.

[0026] FIG. 15. Structure D (SEQ ID NO:18) Native K2S molecule from amino acids 260-527 without modification. This molecule may be further modified in a similar manner as disclosed for structure B in figures 10-13.

[0027] FIG. 16. tPA molecule (SEQ ID NO:19)

TABLE 1. DETECTION OF R-K2S MOLECULE IN PHAGE PREPARATION BY SANDWICH ELISA

Capture antibody	Tracer antibody (conjugated HRP)			
	Anti-tPA		Anti-M13	
	K2S-ϕ	VCSM13[a]	K2S-ϕ	VCSM13
Anti-kringle 2[b]	1.12 ± 0.04[c]	0.12 ± 0.03	1.89 ± 0.02	0.16 ± 0.02
Anti-M13	0.17 ± 0.01	0.14 ± 0.05	1.91 ± 0.02	1.88 ± 0.03

[a] VCSM13 was harvested from XL-1 Blue transformed with pComb3HSS.
[b] Mouse monoclonal anti-kringle 2 (16/B) was used. The other antibodies were prepared from sheep immunoglobulin.
[c] Value is mean of absorbance of each sample which was assayed in triplicate.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0028] The use of the singular or plural in the claims or specification is in no way intended to be limiting and also includes the other form.

[0029] The invention relates to a method for the production of a recombinant DNA-derived tissue plasminogen activator (tPA), a tPA variant, a Kringle 2 Serine protease molecule (K2S) or a K2S variant in prokaryotic cells, wherein said tPA, tPA variant, K2S molecule or K2S variant is secreted extracellularly as an active and correctly folded protein, characterized in that the prokaryotic cell contains and expresses a vector comprising the DNA coding for said tPA, tPA variant, K2S molecule or K2S variant operably linked to the DNA coding for the signal peptide OmpA or a functional derivative thereof.

[0030] Surprisingly, the use of the signal peptide OmpA alone and/ or in combination with the N-terminal amino acids SEGN (SEQ ID NO:9) / SEGNSD (SEQ ID NO:10) translocate the recombinant DNA-derived tPA, tPA variant, K2S molecule or K2S variant to the outer surface and facilitates the release of the functional and active molecule into the culture medium to a greater extent than any other method in the prior art. Before crossing the outer membrane, the recombinant DNA-derived protein is correctly folded according to the method of the present invention. The signal peptide is cleaved off to produce a mature molecule. Surprisingly, the efficiency of signal

peptide removal is very high and leads to correct folding of the recombinant DNA-derived protein.

[0031] Said signal peptide OmpA interacts with SecE and is delivered across the inner membrane by energy generated by SecA, which binds to Sec components (SecE-SecY). SecY forms a secretion pore to dispatch the recombinant DNA-derived protein according to the invention. The space between the outer membrane and inner membrane of Gram-negative bacteria, periplasm, has higher oxidative condition in comparison to the cytoplasmic space. This supports the formation of disulfide bonds and properly folding of the recombinant DNA-derived protein (e.g. K2S) in the periplasm to yield an active molecule. According to the present invention, the signal peptide will be cleaved off to produce a mature molecule. The complex of GspD secretin and GspS lipoprotein on the outer membrane serves as gate channel for secreting the recombinant DNA-derived protein according to the invention to the extracellular medium. This secretion process requires energy, which is generated in cytoplasm by GspE nucleotide-binding protein then transferred to the inner membrane protein (Gsp G-J, F and K-N). GspC transfers the energy to GspD by forming a cross-linker between a set of inner membrane protein (Gsp G-J, F and K-N) and GspD. Before crossing the outer membrane successfully, the recombinant DNA-derived protein is correctly folded.

[0032] Operably linked according to the invention means that the DNA encoding the tPA, tPA variant, K2S molecule or K2S variant (preferably comprising the nucleic acid encoding SEGN or SEGNSD at its N-terminal portion) is cloned in close proximity to the OmpA DNA into the vector in order to achieve expression of the OmpA-tPA, tPA variant, K2S molecule or K2S variant-fusion protein and to direct secretion outside the prokaryotic host cell. Typically, the majority of the tPA, tPA variant, K2S molecule or K2S variant is secreted and can then be purified by appropriate methods such as ammonium sulfate precipitation and/or affinity chromatography and further purification steps. The invention also includes the use of inducers such as IPTG or IPTG in combination with glycerol, the improvement of the

incubation condition and harvesting period to maximize the amount of active protein.

[0033] In a preferred embodiment, said DNA encoding the OmpA signal peptide may be fused to a short peptide characterized by the amino acid sequence SEGN or SEGNSD or the coding nucleic acid sequence TCTGAGGGAAAC (SEQ ID NO:20) or TCTGAGGGAAACAGTGAC (SEQ ID NO:1) and located in the N-terminal portion or at the N-terminal portion of the tPA, tPA variant, K2S molecule or K2S variant. Thus, preferably, said fusion protein comprises OmpA-SEGNSD-tPA, -tPA-variant, -K2S-molecule or -K2S-variant. Even more preferred, said amino acids characterized by SEGN or SEGNSD may be carry a point mutation or may be substituted by a non-natural amino acid. Even more preferred, there may be an amino acid or non-amino acid spacer between OmpA and SEGN or SEGNSD and the tPA, tPA variant, K2S molecule or K2S variant.

[0034] Thus, in a preferred method according to the invention said the prokaryotic cell contains and expresses a vector comprising the DNA coding for said tPA, tPA variant, K2S molecule or K2S variant operably linked to the DNA coding for the signal peptide OmpA which is operably linked to the nucleic acid molecule defined by the sequence TCTGAGGGAAACAGTGAC or a functional derivative thereof.

[0035] The method according to the invention comprises prokaryotic host cells such as, but not limited to *Escherichia coli (E. coli)*, *Bacillus subtilis*, *Streptomyces*, *Pseudomonas, e.g. Pseudomonas putida, Proteus mirabilis, Saccharomyces, Pichia* or *Staphylococcus*, e.g. *Staphylococcus carnosus*. Preferably said host cells according to the invention are Gram-negative bacteria.

[0036] Preferably, a method according to the invention is also characterised in that the prokaryotic cell is *E. coli*. Suitable strains include, but are not limited to *E. coli* XL-1 blue, BL21(DE3), JM109, DH series, TOP10 and HB101. Preferably, a method according to the invention is also characterised in that the following steps are carried out:

a) the DNA encoding the tPA, tPA variant, K2S molecule or K2S variant is amplified by PCR;

b) the PCR product is purified;

c) said PCR product is inserted into a vector comprising the DNA coding for OmpA signal peptide and the DNA coding for gpIII in such a way that said PCR product is operably linked upstream to the DNA coding for the OmpA signal sequence and linked downstream to the DNA coding for gpIII of said vector;

d) that a stop codon is inserted between said tPA, tPA variant, K2S molecule or K2S variant and gpIII;

e) said vector is expressed by the prokaryotic cell

f) the tPA, tPA variant, K2S molecule or K2S variant is purified.

[0037] For step a) according to the invention the choice / design of the primers is important to clone the DNA in the right location and direction of the expression vector (see example 1). Thus, the primers as exemplified in example 1 and figure 4 comprise an important aspect of the present invention. With gp III of step c) gene protein III is meant which is present mainly in phagemid vectors. The stop codon is inserted to avoid transcription of gp III thus eventually leading to secretion of the tPA, tPA variant, K2S molecule or K2S variant of interest. Any suitable method for insertion of the stop codon may be employed such as site-directed mutagenesis (e.g., Weiner MP, Costa GL (1994) PCR Methods Appl 4(3):S131-136; Weiner MP, Costa GL, Schoettlin W, Cline J, Mathur E, Bauer JC (1994) Gene 151(1-2):119-123; see also example 1).

[0038] Any vector may be used in the method according to the invention, preferably said vector is a phagemid vector (see below).

[0039] Preferably, a method according to the invention is also characterised in that the tPA, tPA variant, K2S molecule or K2S variant is selected from human tissue plasminogen activator (tPA, figure 16) or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant therof. Such fragments, allelic variants,

functional variants, variants based on the degenerative nucleic acid code, fusion proteins with an tPA protein according to the invention, chemical derivatives or a glycosylation variant of the tPA proteins according to the invention may include one, several or all of the following domains or subunits or variants thereof:

1. Finger domain (4-50)
2. Growth factor domain (50-87)
3. Kringle 1 domain (87-176)
4. Kringle 2 domain (176-262)
5. Protease domain (276-527)

[0040] The numbering/naming of the domains is according to Genbank accession number GI 137119 or Nature 301 (5897), 214-221 (1983).

[0041] More preferably, a method according to the invention is also characterised in that the tPA, tPA variant, K2S molecule or K2S variant is selected from the Kringle 2 (Barbas, C. F. III and Wagner, J., *et al., Enzymology 8*: 94-103 (1995)) plus Serine protease (Bennett, W. F., *et al., J Biol Chem. 266*:5191-5201 (1991)) K2S variant of human tissue plasminogen activator or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant therof.

[0042] More preferably, a method according to the invention is also characterised in that the vector is a phagemid vector comprising the DNA coding for OmpA signal peptide and the DNA coding for gpIII.

[0043] More preferably, a method according to the invention is also characterised in that the vector is the pComb3HSS phagemid (see also example 1).

[0044] More preferably, a method according to the invention is also characterised in that the DNA sequence comprises or consists of the following DNA sequence encoding OmpA and K2S or a functional variant thereof or a variant due to the degenerate nucleotide code:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCG
CTACCGTGGCCCAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTT

TGGGAATGGGTCAGCCTACCGTGGCACGCACAGCCTCACCGAGTCG
GGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATAGGCAAGG
TTTACACAGCACAGAACCCCAGTGCCCAGGCACTGGGCCTGGGCA
AACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTG
CCACGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGT
GCCCTCCTGCTCCACCTGCGGCCTGAGACAGTACAGCCAGCCTCAG
TTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCCCACCCCT
GGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCGGAGAGC
GGTTCCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCT
GCCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGG
TGATCTTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGC
AGAAATTTGAAGTCGAAAAATACATTGTCCATAAGGAATTCGATGA
TGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGAT
TCGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCC
TTCCCCCGGCGGACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCT
CTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTCGGAG
CGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCA
CATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTGTG
TGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACTTGCACGA
CGCCTGCCAGGGCGATTCGGGAGGCCCCCTGGTGTGTCTGAACGAT
GGCCGCATGACTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTG
GACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACCAACTACCT
AGACTGGATTCGTGACAACATGCGACCG (SEQ ID NO:2)

[0045] More preferably, a method according to the invention is also characterised in that the DNA Sequence of OmpA comprises or consists of the following sequence or a functional variant thereof or a variant due to the degenerate nucleotide code:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCG
CTACCGTGGCCCAGGCGGCC (SEQ ID NO:3).

[0046] Said DNA encodes the following amino acid sequence of OmpA. OmpA thus comprises or consists of a protein characterized by the following

amino acid sequence or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative or a glycosylation variant therof as part of the invention:

MKKTAIAIAVALAGFATVAQAA (SEQ ID NO:21).

[0047] The untranslated region may contain a regulatory element, such as e.g. a transcription initiation unit (promoter) or enhancer. Said promoter may, for example, be a constitutive, inducible or development-controlled promoter. Preferably, without ruling out other known promoters, the constitutive promoters of the human Cytomegalovirus (CMV) and Rous sarcoma virus (RSV), as well as the Simian virus 40 (SV40) and Herpes simplex promoter. Inducible promoters according to the invention comprise antibiotic-resistant promoters, heat-shock promoters, hormone-inducible "Mammary tumour virus promoter" and the metallothioneine promoter. Preferred promotors include the T3 promotor, T7 promotor, Lac/ara1 and Ltet0-1.

[0048] More preferably, a method according to the invention is also characterised in that the DNA of the tPA, tPA variant, K2S molecule or K2S variant is preceeded by a lac promotor and/or a ribosomal binding site such as the Shine-Dalgarno sequence (see also example).

[0049] More preferably, a method according to the invention is also characterised in that the DNA coding for the tPA, tPA variant, K2S molecule or K2S variant is selected from the group of DNA molecules coding for at least 90% of the amino acids 87 – 527, 174 – 527, 180 – 527 or 220 – 527 of the human tissue plasminogen activator protein.

[0050] More preferably, a method according to the invention is also characterised in that the DNA Sequence of K2S comprises or consists of the following sequence:

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACC
GTGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTG
GAATTCCATGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCC
AGTGCCCAGGCACTGGGCCTGGGCAAACATAATTACTGCCGGAATC
CTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAACCGCA

GGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGG
CCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTC
TTCGCCGACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCA
AGCACAGGAGGTCGCCCGGAGAGCGGTTCCTGTGCGGGGGCATAC
TCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTCCAGGAG
AGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATACC
GGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAAT
ACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACAT
TGCGCTGCTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCCAGGAG
AGCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGC
TGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATG
AGGCCTTGTCTCCTTTCTATTCGGAGCGGCTGAAGGAGGCTCATGT
CAGACTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAAC
AGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGAGC
GGCGGGCCCCAGGCAAACTTGCACGACGCCTGCCAGGGCGATTCG
GGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGG
GCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGG
GTGTGTACACAAAGGTTACCAACTACCTAGACTGGATTCGTGACAA
CATGCGACCGTGA (SEQ ID NO:4).

[0051] The present invention also relates to variants of the before-mentioned nucleic acid molecules due to the degenerate code or to fragments therof, nucleic acids which hybridize to said nucleic acids under stringent conditions, allelic or functional variants. The invention also relates to nucleic acids comprising said K2S nucleic acid fused to the nucleic acid encoding another protein molecule.

[0052] Stringent conditions as understood by the skilled person are conditions which select for more than 85 %, preferred more than 90 % homology (Sambrook *et al.* 1989; Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). The hybridisation will be carried out e.g. in 6x SSC/ 5x Denhardt's solution/ 0,1 % SDS (SDS: sodium dodecylsulfate) at 65 °C. The degree of stringency is

decided in the washing step. Thus, for example for a selection of DNA-sequences with approx. 85 % or more homology, the conditions 0,2 x SSC/ 0,01 % SDS/ 65 °C and for a selection of DNA-sequences of approx. 90 % or more homology the conditions 0,1x SSC/ 0,01 % SDS/ 65 °C are suitable. The composition of said reagents is described in Sambrook *et al.* (1989, supra).

[0053] Another important part of the present invention is a variant of human tissue plasminogen activator comprising of or consisting of the Kringle 2 (Barbas, C. F. III and Wagner, J., *et al., Enzymology 8*: 94-103 (1995)) plus Serine protease (Bennett, W. F., *et al., J Biol Chem. 266*:5191-5201 (1991)) (abbreviated K2S) protein or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant therof.

[0054] The numbering/naming of the domains is according to Genbank accession number GI 137119 or Nature 301 (5897), 214-221 (1983), wherein the Kringle 2 domain extends from amino acid 176-262 and the protease domain from 276-527. Thus, according to the invention, a preferred K2S molecule may include amino acids 176-527 including the amino acids between Kringle 2 and the protease (amino acids 263 to 275; exemplified in fig. 8 (structure A)). A K2S molecule according to the invention comprises the minimal part of the Kringle 2 domain and the protease domain still retaining protease activity and fibrin binding activity (measured as exemplified in the description/example). Said K2S molecule according to the invention comprises the amino acids SEGN or SEGNSD in its N-terminal portion (see infra). A preferred K2S molecule does not include amino acids 1 to 3 or 1 to 5 of the tPA molecule. Preferably, a K2S molecule according to the invention has the amino acid Asn at positions 177 and 184, i.e. it does not require the modifications as disclosed in Waldenström for improved producibility with a method according to the invention. Thus, a preferred K2S molecule according to the invention has the native amino acid sequence (no mutation) as opposed to the molecules known from the prior art. Most preferred, said K2S molecule

according to the invention is a molecule characterized by the native amino acid sequence or parts thereof, does neither have amino acids 1 to 3 nor 1 to 5 of tPA and comprises N-terminally the amino acids SEGN or SEGNSD for improved producibility and/or correct folding of the molecule.

[0055] It is essential that the K2S protein according to the invention comprises in its N-terminal portion a peptide characterized by the amino acid sequence SEGN which advantageously allows commercial production with a method as described supra leading to a correctly folded, secreted K2S protein. Said 4 amino acids characterized by SEGN may have one or several amino acids more N-terminal, however said amino acids have to be located in the N-terminal portion as opposed to the C-terminal portion. Most preferably, said amino acids are located at the N-terminal portion. Preferably, the amino acids characterized by SEGN may be carry a point mutation or may be substituted by a non-natural amino acid.

[0056] Thus, in another important embodiment the invention relates to a K2S protein characterized in that it comprises the amino acids defined by the sequence SEGN or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant therof.

[0057] Such fragments are exemplified e.g. in figure 10 (Structure B-1) and figure 11 (Structure B-2) extending from amino acids 193-527. Structure B-1 has the native amino acid Cys in position 261, wherein in B-2 the amino acid is substituted by Ser. Further fragments according to the invention comprising the amino acids 220-527 (fig. 14, structure C) or comprising the amino acids 260-527 (fig. 15, structure D) may be modified according to the invention by addition of the amino acids SEGN and/or substitution of Cys-261 by Ser. The artisan can determine the minimal length of a K2S molecule according to the invention in order to retain its biological function and generate a K2S molecule with improved producibility and/or correct folding by adding the amino acids SEGN in the N-terminal portion. Thus, another preferred

embodiment is said minimal K2S molecule with SEGN at its N-terminal portion.

[0058] In another important embodiment the invention relates to a K2S protein characterized in that it comprises the amino acids defined by the sequence SEGNSD or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant therof. Such fragments are exemplified e.g. in figure 12 (Structure B-3) and figure 13 (Structure B-4) extending from amino acids 191-527. Structure B-3 has the native amino acid Cys in position 261, wherein in B-4 the amino acid is substituted by Ser. Further fragments according to the invention comprising the amino acids 220-527 (fig. 14, structure C) or comprising the amino acids 260-527 (fig. 15, structure D) may be modified according to the invention by addition of the amino acids SEGNSD and/or substitution of Cys-261 by Ser. The artisan can determine the minimal length of a K2S molecule according to the invention in order to retain its biological function and generate a K2S molecule with improved producibility and/or correct folding by adding the amino acids SEGNSD in the N-terminal portion. Thus, another preferred embodiment is said minimal K2S molecule with SEGNSD at its N-terminal portion.

[0059] Another more preferred embodiment of the present invention relates to a K2S protein comprising a protein characterized by the following amino acid sequence or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative or a glycosylation variant therof:

SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSA
QALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLR
QYSQPQFRIKGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWI
LSAAHCFQERFPPHHLTVILGRTYRVVPGEEEQKFEVEKYIVHKEFDD
DTYDNDIALLQLKSDSSRCAQESSVVRTVCLPPADLQLPDWTECELSG
YGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAGD
TRSGGPQANLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKD
VPGVYTKVTNYLDWIRDNMRP* (SEQ ID NO:11).

[0060] According to the invention, * means STOP (i.e. encoded by a stop codon). This K2S molecule is exemplified in figure 8.

[0061] One variant of the K2S molecule according to the invention relates to a fusion protein of K2S being fused to another protein molecule.

[0062] Another more preferred embodiment of the present invention relates to a K2S protein consisting of a protein characterized by the following amino acid sequence:

SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSA
QALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLR
QYSQPQFRIKGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWI
LSAAHCFQERFPPHHLTVILGRTYRVVPGEEEQKFEVEKYIVHKEFDD
DTYDNDIALLQLKSDSSRCAQESSVVRTVCLPPADLQLPDWTECELSG
YGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAGD
TRSGGPQANLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKD
VPGVYTKVTNYLDWIRDNMRP* (SEQ ID NO:11).

[0063] Said K2S molecules may be encoded by a DNA molecule as described supra.

[0064] Another important aspect of the invention relates to a DNA molecule characterized in that it is coding for:

a) the OmpA protein or a functional derivative therof operably linked to

b) a DNA molecule coding for a polypeptide containing the kringle 2 domain and the serine protease domain of tissue plasminogen activator protein.

[0065] More preferably, a DNA molecule according to the invention is also characterised in that the DNA sequence comprises or consists of the following DNA sequence encoding OmpA and K2S or a functional variant thereof or a variant due to the degenerate nucleotide code:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCG
CTACCGTGGCCCAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTT
TGGGAATGGGTCAGCCTACCGTGGCACGCACAGCCTCACCGAGTCG

GGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATAGGCAAGG
TTTACACAGCACAGAACCCCAGTGCCCAGGCACTGGGCCTGGGCA
AACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTG
CCACGTGCTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGT
GCCCTCCTGCTCCACCTGCGGCCTGAGACAGTACAGCCAGCCTCAG
TTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCCCACCCCT
GGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCGGAGAGC
GGTTCCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCT
GCCGCCCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGG
TGATCTTGGGCAGAACATACCGGGTGGTCCCTGGCGAGGAGGAGC
AGAAATTTGAAGTCGAAAAATACATTGTCCATAAGGAATTCGATGA
TGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGAT
TCGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCC
TTCCCCCGGCGGACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCT
CTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTCGGAG
CGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCA
CATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTGTG
TGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACTTGCACGA
CGCCTGCCAGGGCGATTCGGGAGGCCCCCTGGTGTGTCTGAACGAT
GGCCGCATGACTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTG
GACAGAAGGATGTCCCGGGTGTGTACACAAAGGTTACCAACTACCT
AGACTGGATTCGTGACAACATGCGACCG (SEQ ID No:2)

[0066] Said DNA molecule encodes the following fusion protein of OmpA and K2S. Said fusion protein of OmpA and K2S characterised in that it comprises or consists of a protein characterized by the following amino acid sequence or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative or a glycosylation variant therof forms an important part of the present invention:

MKKTAIAIAVALAGFATVAQAASEGNSDCYFGNGSAYRGTHSLTESG
ASCLPWNSMILIGKVYTAQNPSAQALGLGKHNYCRNPDGDAKPWCH
VLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIASHPWQA

AIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHHLTVILGRTY
RVVPGEEEQKFEVEKYIVHKEFDDDTYDNDIALLQLKSDSSRCAQESS
VVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYP
SSRCTSQHLLNRTVTDNMLCAGDTRSGGPQANLHDACQGDSGGPLVC
LNDGRMTLVGIISWGLGCGQKDVPGVYTKVTNYLDWIRDNMRPG
(SEQ ID NO:8)

[0067] Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 87 – 527 of the human tissue plasminogen activator protein (numbering used herein as GI 137119 or Nature 301 (5897), 214-221 (1983).

[0068] Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 174 – 527 of the human tissue plasminogen activator protein.

[0069] Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 180 – 527 of the human tissue plasminogen activator protein.

[0070] Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 220 – 527 of the human tissue plasminogen activator protein.

[0071] Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence a) is hybridizing under stringent conditions to the following sequence:
ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCG
CTACCGTGGCCCAGGCGGCC (SEQ ID NO:3).

[0072] Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence a) consists of the following sequence:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCG
CTACCGTGGCCCAGGCGGCC (SEQ ID NO:3).

[0073] Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is hybridizing under stringent conditions to the following sequence:

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACC
GTGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTG
GAATTCCATGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCC
AGTGCCCAGGCACTGGGCCTGGGCAAACATAATTACTGCCGGAATC
CTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAACCGCA
GGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGG
CCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTC
TTCGCCGACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCA
AGCACAGGAGGTCGCCCGGAGAGCGGTTCCTGTGCGGGGGCATAC
TCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTCCAGGAG
AGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATACC
GGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAAT
ACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACAT
TGCGCTGCTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCCAGGAG
AGCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGC
TGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATG
AGGCCTTGTCTCCTTTCTATTCGGAGCGGCTGAAGGAGGCTCATGT
CAGACTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAAC
AGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGAGC
GGCGGGCCCCAGGCAAACTTGCACGACGCCTGCCAGGGCGATTCG
GGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGG
GCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGG
GTGTGTACACAAAGGTTACCAACTACCTAGACTGGATTCGTGACAA
CATGCGACCGTGA (SEQ ID NO:4).

[0074] Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) consists of the following sequence:

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACC
GTGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTG
GAATTCCATGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCC
AGTGCCCAGGCACTGGGCCTGGGCAAACATAATTACTGCCGGAATC
CTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAACCGCA
GGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGG
CCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTC
TTCGCCGACATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCA
AGCACAGGAGGTCGCCCGGAGAGCGGTTCCTGTGCGGGGGCATAC
TCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTCCAGGAG
AGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATACC
GGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAAT
ACATTGTCCATAAGGAATTCGATGATGACACTTACGACAATGACAT
TGCGCTGCTGCAGCTGAAATCGGATTCGTCCCGCTGTGCCCAGGAG
AGCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGC
TGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATG
AGGCCTTGTCTCCTTTCTATTCGGAGCGGCTGAAGGAGGCTCATGT
CAGACTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAAC
AGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGAGC
GGCGGGCCCCAGGCAAACTTGCACGACGCCTGCCAGGGCGATTCG
GGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGG
GCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGG
GTGTGTACACAAAGGTTACCAACTACCTAGACTGGATTCGTGACAA
CATGCGACCGTGA (SEQ ID NO:4).

[0075] Another preferred embodiment of the invention relates to a vector containing a DNA sequence according to the invention.

[0076] Another preferred embodiment of the invention relates to a vector according to the invention, wherein said DNA sequence is preceeded by a lac

promoter and a ribosomal binding site. Suitable vectors according to the invention include, but are not limited to viral vectors such as e.g. Vaccinia, Semliki-Forest-Virus and Adenovirus, phagemid vectors and the like. Preferred are vectors which can be advantageously used in *E. coli*, but also in any other prokaryotic host such as pPROTet.E, pPROLar.A, members of the pBAD family, pSE family, pQE family and pCAL.

[0077] Another preferred embodiment of the invention relates to the vector pComb3HSS containing a DNA according to the invention, wherein the expression of the gp III protein is suppressed or inhibited by deleting the DNA molecule encoding said gp III protein or by a stop codon between the gene coding for a polypeptide containing the kringle 2 domain and the serine protease domain of tissue plasminogen activator protein and the protein III gene.

[0078] Another important aspect of the present invention relates to a prokaryotic host cell comprising a DNA molecule according to the invention.

[0079] Another important aspect of the present invention relates to a prokaryotic host cell comprising a vector according to the invention.

[0080] Another important aspect of the present invention relates to an *E. coli* host cell comprising a DNA molecule according to the invention.

[0081] Another important aspect of the present invention relates to a *E. coli* host cell comprising a vector according to the invention.

[0082] Yet another important aspect of the present invention is the use of a DNA molecule according to the invention or of a vector according to the invention or a host cell according to the invention in a method for the production of a polypeptide having the activity of tissue plasminogen activator.

[0083] Yet another important aspect of the present invention is the use according the invention as described above, wherein said method is a method according to the invention.

[0084] Another very important aspect is a pharmaceutical composition comprising a substance obtainable by a method according to the invention and

pharmaceutically acceptable excipients and carriers. An example for said substance is the K2S molecule described supra. The term "pharmaceutically acceptable carrier" as used herein refers to conventional pharmaceutic excipients or additives used in the pharmaceutical manufacturing art. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients (see also e.g. Remington's Pharmaceutical Sciences (1990, 18th ed. Mack Publ., Easton)). Said pharmaceutical composition according to the invention can be advantageously administered intravenously as a bolus, e.g. as a single bolus for 5 to 10 seconds intravenously.

[0085] The invention further relates to the use of substances obtainable by a method according to the invention in the manufacture of a medicament in the treatment of stroke, cardiac infarction, acute myocardial infarction, pulmonary embolism, any artery occlusion such as coronary artery occlusion, intracranial artery occlusion (e.g. arteries supplying the brain), peripherally occluded arteries, deep vein thrombosis or related diseases associated with unwanted blood clotting.

[0086] The following example is intended to aid the understanding of the invention and should in no way be regarded as limiting the scope of the invention.

EXAMPLES

EXAMPLE 1

Materials And Methods

Primer design

[0087] In order to amplify a specific part of tPA gene, a pair of primers SK2/174

[5' GAGGAGGAGGTGGCCCAGGCGGCCTCTGAGGGAAACAGTGAC 3'] (SEQ ID NO:22) and ASSP

[5' GAGGAGGAGCTGGCCGGCCTGGCCCGGTCGCATGTTGTCACG 3'] (SEQ ID NO:23) were synthesized (Life Technologies, Grand Island, NY). These primers were designed based on the human tPA gene retrieved from NCBI databases (g137119). They were synthesized with Sfi I end cloning sites (underlined) in such a way that the reading frame from the ATG of the gpIII gene in phagemid vector, pComb3HSS, will be maintained throughout the inserted sequence.

[0088] Another primer set for site-directed mutagenesis was designed to anneal at the sequence situated between K2S gene and gene III in pComb3H-K2S. The sequence of primers with mutation bases (underlined) for generating a new stop codon were MSTPA

[5' ACATGCGACCGTGACAGGCCGGCCAG 3'] (SEQ ID NO:24) and MASTPA

[5' CTGGCCGGCCTGTCACGGTCGCATGT 3'] (SEQ ID NO:25).

[0089] Amplification of K2S gene by PCR. One μg sK2/174 and ASSP primers together with 50 ng of p51-3 template (obtained from Dr. Hiroshi Sasaki, Fujisawa Pharmaceutical, Japan) were suspended in 100 μl PCR mixture. An amount of 2.5 U Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN) was finally added to the solution. The titrated amplification condition was initiated with jump start at 85°C for 4 min, then denaturation at 95°C for 50 sec, annealing at 42°C for 50 sec, extension at 72°C for 1.5 min. Thirty five rounds were repeatedly performed. The mixture was further incubated at 72°C for 10 min. The amplified product of 1110 bp was subsequently purified by QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The correctness of purified product was confirmed by restriction enzymes.

[0090] Construction of phagemid expressing K2S. The purified PCR product of K2S and pComb3HSS phagemid (kindly provided by Dr. Carlos F. Barbas, Scripps Institute, USA) were digested with Sfi I (Roche Molecular

Biochemicals, Indianapolis, IN) to prepare specific cohesive cloning sites. Four μg of the purified PCR product was digested with 60 U of Sfi I at 50°C for 18 h. For pComb3HSS, 20 μg of phagemid vectors were treated with 100 U of Sfi I. Digested products of purified PCR product of K2S and pComb3HSS (~3300 bp) were subsequently gel-purified by the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). T4 ligase (Roche Molecular Biochemicals, Indianapolis, IN) of 5 U were introduced to the mixture of 0.7 μg of purified Sfi I-digested pComb3HSS and 0.9 μg of purified Sfi I-digested PCR product. Ligation reaction was incubated at 30°C for 18 h. The newly constructed phagemid was named pComb3H-K2S.

[0091] Transformation of *E. coli* XL-1 Blue. Two hundred μl of $CaCl_2$ competent *E. coli* XL-1 Blue (Stratagene, La Jolla, CA) were transformed with 70 ng of ligated or mutated product. The transformed cells were propagated by spreading on LB agar containing 100 μg/ml ampicillin and 10 μg/ml tetracycline (Sigma, Saint Louis, MO). After cultivation at 37°C for 18 h several antibiotic resistant colonies were selected for plasmid minipreps by using the alkaline lysis method. Each purified plasmid was subjected to Sfi I restriction site analysis. A transformant harboring plasmid with the correct Sfi I restriction site(s) was subsequently propagated for 18 h at 37°C in 100 ml LB broth with ampicillin 100 μg/ml and tetracycline 10 μg/ml. A plasmid maxiprep was performed using the QIAGEN Plasmid Maxi Kit (QIAGEN, Hilden, Germany). The purified plasmid was reexamined for specific restriction sites by Sfi I and sequenced by AmpliTaq DNA Polymerase Terminator Cycle Sequencing Kit (The Perkin-Elmer Corporation, Forster City, CA).

[0092] Site-directed mutagenesis of pComb3H-K2S. 10 ng of pComb3H-K2S template were mixed with 125 ng of MSTPA and MASTPA primers. PfuTurbo DNA polymerase (Stratagene, LA Jolla, CA) of 2.5 U was added to the mixture for cycle amplification. The reaction started with one round of 95°C for 30 sec. Then it was followed by 16 rounds consisting of 95°C for 30 sec, 55°C for 1 min, and 68°C for 9 min. The reaction tube was subsequently

placed on ice for 2 min. In order to destroy the template strands, 10 U of Dpn I restriction enzyme (Stratagene, LA Jolla, CA) were added to the amplification reaction and incubated for 1 h at 37°C. This synthesized product (MpComb3H-K2S) was further used to transform *E. coli* XL-1 Blue.

[0093] Preparation of phage-display recombinant-K2S. After pComb3H-K2S was transformed to XL-1 Blue, the phage display technique was performed. A clone of pComb3H-K2S transformed *E. coli* XL-1 Blue was propagated in 10 ml super broth containing ampicillin 100 μg/ml and tetracycline 10 μg/ml at 37°C until the O.D. [600 nm] of 1.5 was reached. The bacterial culture was subsequently propagated in 100 ml of the same medium and culture for 2 h. An amount of 10^{12} pfu of VCSM13 helper phage (Stratagene, La Jolla, CA) was used to infect the transformed *E. coli* XL-1 Blue. After 3 h incubation, kanamycin at a final concentration of 70 μg/ml final concentration was added to culture. The culture was left shaking (200 RPM) for 18 h at 37°C. Bacteriophages which harbored K2S on gpIII (K2S-ϕ) were then harvested by adding 4% w/v PEG MW 8000 (Sigma, Saint Louis, MO) and 3% w/v NaCl. Finally, the harvested phage was resuspended in 2 ml PBS pH 7.4. The phage number was determined by infecting *E. coli* XL-1 Blue. The colony-forming unit per milliliter (cfu/ml) was calculated as described previously (Lobel, L. I., *et al., Endocrinology. 138*:1232-1239 (1997)).

[0094] Expression of recombinant-K2S in shaker flasks. MpComb3H-K2S transformed *E. coli* XL-1 Blue was cultivated in 100 ml super broth (3% w/v tryptone, 2% w/v yeast extract and 1% w/v MOPS) at pH 7.0 in the presence of ampicillin (100 μg/ml) at 37°C until an O.D. [600 nm] of 0.8 was reached. Subsequently, the protein synthesis was induced by 1 mM of IPTG (Promega, Madison, WI). The bacteria were further cultured shaking (200 RPM) for 6 h at 30°C. The culture supernatant was collected and precipitated with 55% saturated ammonium sulfate (Soeda, S., *et al., Life Sci. 39*:1317-1324 (1986)). The precipitate was reconstituted with PBS, pH 7.2, and dialysed in the same buffer solution at 4°C for 18 h. Periplasmic proteins from bacterial cells were

extracted by using a chloroform shock as previously described by Ames *et al.* (Ames, G. F., *et al., J. Bacteriol. 160*:1181-1183 (1984)).

[0095] Immunoassay quantification of recombinant-K2S. In order to detect r-K2S, solid phase was coated with monoclonal anti-kringle 2 domain (16/B) (generously provided by Dr. Ute Zacharias, Central Institute of Molecular Biology, Berlin-Buch, Germany). The standard ELISA washing and blocking processes were preformed. Fifty µl of 10^{11} cfu/ml of K2S-ϕ or secretory r-K2S were added into each anti-kringle 2 coated well. Antigen-antibody detection was carried out as follows. Either sheep anti-M13 conjugated HRP (Pharmacia Biotech, Uppsala, Sweden) or sheep anti-tPA conjugated HRP (Cedarlane, Ontario, Canada), was added to each reaction well after the washing step. The substrate TMB was subjected to every well and the reaction was finally ceased with H_2SO_4 solution after 30 min incubation. The standard melanoma tPA 86/670 (National Institute for Biological Standards and Control, Hertfordshine, UK) was used as positive control.

[0096] Amidolytic activity assay. A test kit for the detection of tPA amidolytic activity was purchased from Chromogenix (Molndal, Sweden). The substrate mixture containing plasminogen and S-2251 was used to determine serine protease enzymatic activity. The dilution of 10^{-2} of each ammonium precipitated sample was assayed with and without stimulator, human fibrinogen fragments. The assay procedure was according to the COASET t-PA manual.

[0097] SDS-PAGE and immunoblotting. The dialysed precipitate-product from culture supernatant was further concentrated 10 fold with centricon 10 (AMICON , Beverly, MA). The concentrated sample was subjected to protein separation by SDS-PAGE, 15% resolving gel, in the reducing buffer followed by electroblotting to nitrocellulose. The nitrocellulose was then blocked with 4% skimmed milk for 2 hr. In order to detect r-K2S, a proper dilution of sheep anti-tPA conjugated HRP was applied to the nitrocellulose. The immunoreactive band was visualized by a sensitive detection system, Amplified Opti-4CN kit (BIORAD, Hercules, CA).

[0098] Copolymerized plasminogen polyacrylamide gel electrophoresis. An 11% resolving polyacrylamide gel was copolymerized with plasminogen and gelatin as previously described by Heussen *et al.* (Heussen, C. and Dowdle, E.B., *Anal. Biochem. 102*:196-202 (1980)). The stacking gel was prepared as 4 % concentration without plasminogen and gelatin. Electrophoresis was performed at 4°C at a constant current of 8 mA. The residual SDS in gel slab was removed after gentle shaking at room temperature for 1h in 2.5% Triton X-100. Then the gel slab was incubated in 0.1 M glycine-NaOH, pH 8.3, for 5 h at 37°C. Finally, the gel slab was stained and destained by standard Coomassie brilliant blue (R-250) dying system. The location of the peptide harboring enzymatic activity was not stained by dye in contrast to blue-paint background.

RESULTS

[0100] Construction of K2S gene carrying vector. From the vector p51-3 we amplified the kringle 2 plus ther serine protease portion of tPA (Ser^{174} in kringle 2 domain to Pro^{527} in the serine protease) using primers SK2/174 and ASSP. The amplified 1110 bp product was demonstrated by agarose gel electrophoresis (Fig. 1, lane 2) and was inserted into pComb3HSS phagemid by double Sfi I cleavage sites on 5′ and 3′ ends in the correct reading frame. Thus a new vector, pComb3H-K2S, harboring the K2S was generated. In this vector K2S is flanked upstream by the OmpA signal sequence and downstream by gpIII. The correct insertion of K2S was verified both by restriction analysis with Sfi I (Fig. 2, lane 3), PCR-anaysis (demonstration of a single band at 1110 bp), and DNA sequencing. The schematic diagram of pComb3H-K2S map is given in Fig. 3.

[0101] Phage-displayed r-K2S. VCSM13 filamentous phage was used to infect pComb3H-K2S transformed *E. coli* XL-1 Blue, X[K2S]. VCSM13 was propagated and incorporated the K2S-gpIII fusion protein during the viral packaging processes. The harvested recombinant phage (K2S-ϕ) gave a

concentration of 5.4×10^{11} cfu/ml determined by reinfecting *E. coli* XL-1 Blue with PEG-precipitated phages. These recombinant phage particles were verified for the expression of r-K2S by sandwich ELISA. The phage-bound heterologous K2S protein was recognized by the monoclonal anti-kringle 2 antibody (16/B) by using sheep anti-tPA conjugated HRP antibody detection system. The absorbance of this assay was 1.12 ± 0.03 (Table 1). The amount of K2S detectable on 10^{12} phage particles is equal to 336 ng of protein in relation to the standard melanoma tPA. In order to corroborate that K2S-gpIII fusion protein was associated with phage particles, sheep anti-tPA conjugated HRP antibody was substituted by sheep anti-M13 antibody conjugated HRP. This immuno-reaction exhibited an absorbance of 1.89 ± 0.07 (Table 1). In contrast, if the capture antibody was sheep anti-M13 antibody, extremely low K2S was observed with sheep anti-tPA antibody conjugated HRP; the absorbance was only 0.17 ± 0.01 (Table 1). This suggested that only a minority of purified phage particles carried K2S-gpIII fusion protein. VCSM13 prepared from non-transformed XL-1 Blue was used as a negative control.

[0102] Construction of MpComb3H-K2S. We generated a stop codon between K2S and gpIII in pComb3H-K2S with the aid of the mutagenic primers (MSTPA and MASTPA) (Fig. 4). In order to enrich the newly synthesized and mutated MpComb3H-K2S, the cycle amplification mixture was thoroughly digested with Dpn I to degrade the old dam methylated pComb3H-K2S template (Dpn I prefers dam methylated DNA). After transforming of *E. coli* XL-1 Blue with MpComb3H-K2S, a transformant XM[K2S] was selected for further study. As a consequence of bp substitution, one Sfi I cleavage site close to the 3' end of K2S gene was lost after site-directed mutagenesis. A linear version of Sfi I cleaved MpComb3H-K2S was observed at 4319 bp without the appearance of inserted K2S gene fragment (Fig. 5, lane 3). Thus, the K2S gene encoding by MpComb3H-K2S was expressed in non-gpIII fusion form in XM[K2S].

[0103] Expression and purification of K2S. K2S expression in XM[K2S] was induced by IPTG. r-K2S was detectable by using ELISA both in the periplasmic space and in the culture supernatant. The amount of the heterologous protein in each preparation was determined by sandwich ELISA and related to the standard tPA. From 100 ml of the bacterial culture in shaker flask with the O.D. [600 nm] of 50, the periplasmic fraction yielded 1.38 μg of r-K2S (approximately 32%) whereas 2.96 μg of r-K2S (approximately 68%) was obtained in the ammonium precipitated culture supernatant. Sandwich ELISA was used to verify the PEG precipitated phage from VCSM13 infected XM[K2S]. No r-K2S captured by monoclonal anti-kringle 2 antibody was detected by anti-M13 conjugated HRP, indicating that K2S is not presented on the phage particles if gpIII is missing.

[0104] Amidolytic activity measurement. If serine protease domain is present in the sample, plasminogen will be converted to plasmin. The produced plasmin will further digest the S-2251 substrate to a colour product, p-nitroaniline, which has a maximum absorbance at 405 nm. The specific activity of the recombinant product is in accordance with the absorbance. The fibrinogen-dependent enzymatic activity of each sample i.e. K2S-ϕ, periplasmic r-K2S or culture supernatant r-K2S, was evaluated and compared. Both K2S-ϕ and periplasmic r-K2S illustrated notably low enzymatic activity, which was below the sensitivity of the test (0.25 IU/ml). The culture supernatant r-K2S gave the fibrinogen-dependent enzymatic activity of 7 IU/ml. Thus, from 100 ml culture we obtained a total of 700 IU enzymatic activity. Without fibrinogen no enzymatic activity of the r-K2S purified from culture supernatant was observed - whereas standard melanoma tPA showed some activity.

[0105] Demonstration of recombinant protein by immunoblotting. Partially purified K2S from culture supernatant of XM[K2S] revealed a molecular mass of 39 kDa by using sheep anti-tPA antibodies (Fig. 6). The negative control, partially purified culture supernatant of non-transformed XL1-Blue, contained no reactive band with a similar size.

[0106] Localization of active enzyme by PAGE. The plasminogen has been copolymerized and immobilized with gelatin in the polyacrylamide gel prior to electrophoresis. The ammonium sulfate precipitated culture supernatants of *E. coli* XL-1 Blue, *E. coli* XL-1 Blue transformed with pComb3HSS and XM[K2S] were analyzed (Fig. 7). All samples were processed in non-reducing condition to preserve the correct conformation and activity of the serine protease domain. Transparent areas of serine protease digested plasminogen were observed only in the ammonium sulfate precipitated culture supernatants of XM[K2S] at 34 and 37 kDa postions. The other samples gave no clearing zones. The positive control lane of standard melanoma tPA also demonstrated enzymatic activity at 66 and 72 kDa positions.